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FOREWORD

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INTRODUCTION

Evidence has accumulated to support the "angiogenesis switch" hypothesis in the development of cancers, including breast cancer (1, 2). Briefly, it has been shown that in-situ carcinomas may exist for months or years without neovascularization, and as a result, their volumes remain a few cubic millimeters. Some tumor cells then undergo an "angiogenic switch" to change from non-angiogenic to angiogenic. New blood vessels are recruited, which support the growth of both angiogenic and non-angiogenic tumor cells. The mechanism of angiogenesis regulation is yet to be fully understood. It is becoming clear, however, that the initiation or termination of the process is controlled by a balance between positive and negative regulators of angiogenesis. A number of angiogenic factors, often markedly upregulated in tumor tissues, have been described, including several well-studied members of the fibroblast growth factor family, such as FGF-1 (3), FGF-2 (4), and those of the vascular endothelial cell growth factor family (VEGF) (5), as well as the receptors of these growth factors (6-9). Several inhibitors of angiogenesis have also been reported, including thrombospondin (10), angiostatin (11), endostatin (12), platelet factor-4 (13), and the proliferin-related protein (14).

Angiopoietins are a novel family of cytokines that take part in the regulation of angiogenesis (15, 16). Angiopoietin-1 (Ang-1), which is not a mitogenic factor for cultured endothelial cells, has been shown to be able to induce phosphorylation of the endothelial cell specific receptor tyrosine kinase TIE-2 (15). Null mutation analysis (knock-out) of Ang-1 or TIE-2 indicates that this receptor-ligand system plays an essential role in the development of capillary vessels and the structural integration of pericytes and endothelial cells (17). Briefly, in the Tie-2 knocked-out mouse embryos, endothelial cells are present in normal numbers and are assembled into vessels; however, those vessels are lacking branches and missing pericytes that normally encapsulate mature vessels. A similar phenotype is observed for Ang-1 null mutation (18). These findings led to suggestions that the binding of Ang-1 to endothelial cell-surface TIE-2 induces the release of certain factors that facilitate the recruitment of perivascular cells such as smooth muscle cells and myocardiocytes, and maintain the association of these pericytes with endothelial cells, solidifying and stabilizing the neovasculature (19).

BODY

The goal of my research proposal was to evaluate TIE-2 for its ability to serve as such a target. I hypothesized that abrogation of TIE-2 expression or function may critically damage tumor vasculature, and additionally, that TIE-2 may be used as a specific port of entry to introduce cytotoxic agents into endothelial cells undergoing angiogenesis. We proposed 1) to overexpress a secreted form of the extracellular domain of TIE-2 in breast cancer cells and examine the tumorigenic and metastatic potential of the transfected cells implanted in the mammary fat pads of nude mice, and 2) to prepare a recombinant toxin made of the receptor binding domain of the TIE-2 ligand joined with a truncated form of *Pseudomonas* exotoxin that lacks the ability to bind to cells by itself, then examine the fusion protein for anticancer activity in the breast cancer xenograft model. It was hoped that the outcome of the proposed study may lend insights into tumor vascular biology, and provide novel antiangiogenic agents of potential therapeutic value.

Preparation of Ang-1-PE toxin: The receptor binding domain of Ang-1, R284-F498, was subcloned and inserted into a plasmid that contains the PE toxin, between restriction endonuclease Hind III and Kpn I recognition sites. The sequence of Ang-1-PE in the plasmid was confirmed by complete sequencing. The plasmid was then used to transform *E. coli* strain BL21-lamda-DE3. The expression of Ang-1-PE in the transformed cells was inducible by IPTG. When subjected to Western blotting analysis using an anti-PE antibody, a unique band corresponding to a protein of MW 60 kD was identified, as expected for the Ang-1-PE fusion protein. Progress is underway to purify the fusion protein in order to assess its activity. An endothelial cell-specific cell-killing activity is expected.

Investigation on the function of Ang-1/Tie-2 system: We have investigated the role of Ang-1 in tumor angiogenesis and found that the effect of Ang-1 appears to be proangiogenic (20). We have utilized a number of complementary approaches to elucidate a potential pro-angiogenic role for Ang-1. Firstly we have analyzed the expression of Ang-1 in 19 breast cancer cell lines by RT-PCR, and in clinical breast cancer specimens using in-situ hybridization to Ang-1. Secondly we have assessed the angiogenic effect of Ang-1 on an endothelial cell tubule forming assay. Finally we have analyzed the effect of transfection of a non-tumorigenic Chinese hamster ovary (CHO) cell line with Ang-1 on tumor formation in athymic mice. These results are presented below.

The expression of ANG-1 in breast cancer cell lines and clinical specimens: Messenger RNA from 19 breast cancer cell lines was prepared and analyzed with probes for ANG-1 and GAPDH as a loading control. (Figure 1) The PCR products were subjected to electrophoresis on a 2% agarose gel, visualized by ethidium bromide staining. The gel was transferred to a nitrocellulose membrane and probed with a ³²P-labeled oligonucleotide primer to a sequence of Ang-1 that lies between the PCR primers. Specific signal was seen in 8 out of 19 lines.

Samples of clinical breast cancer specimens and normal breast tissue were collected prospectively, frozen on dry ice and ethanol and stored at -70 C. Sections were analyzed both for Tie-2 expression and Ang-1 expression. Tie-2 expression was assessed by immunohistochemistry using a monoclonal antibody to TIE-2. Excellent co-localization between TIE-2 expression and Von Willebrand Factor was seen on stromal vasculature within tumour samples which confirmed recently published data that TIE-2 is up-regulated in areas of tumor vascularization (21). However significant TIE-2 expression was also seen in normal breast tissues and other normal tissues. We also analyzed the expression of Ang-1 in the same tissues by in-situ hybridization using a ³⁵S-labeled RNA probe. We demonstrated that in about 25% of interpretable samples signal to ANG-1 was visualized over breast cancer cells in the vicinity of areas of tumor neo-vascularization as visualized by either TIE-2 or Von Willebrand factor (Figure 3). Although, in interpretable sections, the signal to ANG-1 is quite strong, the tissue morphology is not infrequently quite poor, which has presented problems in the accrual of

data from a significant proportion of the samples collected. Nevertheless when this problem is addressed we anticipate demonstrating up-regulation of expression in between 25 and 40% of cases, in keeping with data from the cell lines.

Angiogenic Activity of Ang-1 in endothelial tubule formation assays: We confirmed other workers findings that recombinant Ang-1 protein does not induce endothelial proliferation (data not shown). We therefore assessed the ability of Ang-1 to induce the formation of tubule like structures by adult bovine aortic endothelial (ABAE) cells seeded on a gel of type 1collagen. (Figure 4) We could demonstrate a moderate tubule forming ability of Ang-1 in this assay equaling approximately 50% of the tubule length induced by a saturating dose of the positive control, basic fibroblast growth factor.

Transfection of Ang-1 into CHO cells induces tumorigenicity: We have found that constitutive over-expression of a secreted form of Ang-1 under a HTLV-1 promoter in non-tumorigenic Chinese hamster ovary (CHO) cells converted these cells to a tumorigenic phenotype. This transfection did not result in any alteration in the in-vitro growth characteristics of the CHO cells as compared to the vector transfected cells and both cell lines, though immortalized displayed contact inhibition upon reaching confluence. However, when injected subcutaneously into athymic nude mice, Ang-1-overexpressing cells rapidly formed vascular tumors while the vector-transfected cells were uniformly non-tumorigenic (Fig. 5).

The role of Ang-1 in tumor angiogenesis may be complicated and diverse. There are two plausible directions to investigate the function of Ang-1 in tumor angiogenesis: 1) Ang-1 acts on the endothelial cells of the existing vasculature in the surroundings to induce morphogenesis of blood vessels such as intussusceptive blood vessel division, a proangiogenic effect; 2) Ang-1 induces the endothelial cells to produce angiogenic factors to promote the angiogenic switch necessary for tumorigenesis. The latter effect may include the recruitment of perivascular cells to the neovessels, which stabilizes the vessels and decreases permeability.

Recommendations in Relation to the Statement of Work: In the Statement of Work, the construction of the Ang-1-PE fusion toxin was initially designated as the second year's work (Aim 2). We focused on this work in the first year, however, because it may give rise to an anticancer agent of therapeutic value. In the meantime, we began to investigate the role of Ang-1 in breast cancer. This is directly related to proposed goal of this research project: to evaluate the possibility of using Tie-2 as the target of new antiangiogenic and anticancer drugs. In the second year of the three-year funding period, we will continue to evaluate the activity of the Ang-1-PE fusion toxin. We expect to complete all of the work related to Aim 2. At the same time, we will begin to work on Aim 1: to determine whether overexpressing the extracellular domain of Tie-2 in MB231 breast cancer cells would lead to a decreased tumorigenicity of the cancer cells.

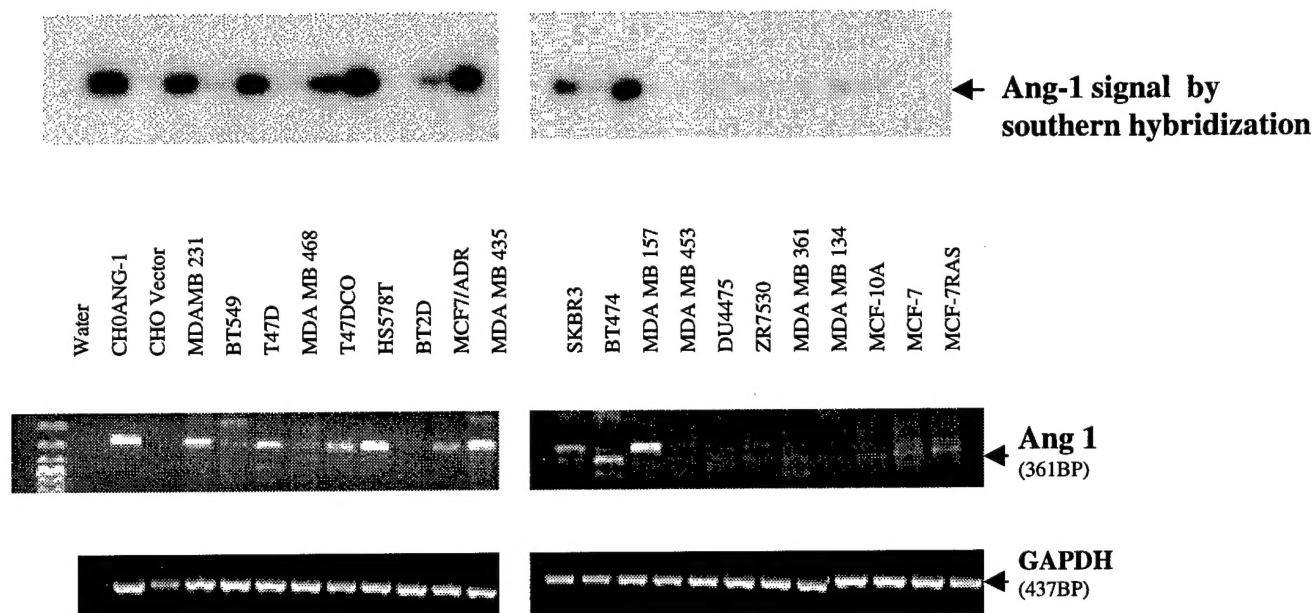
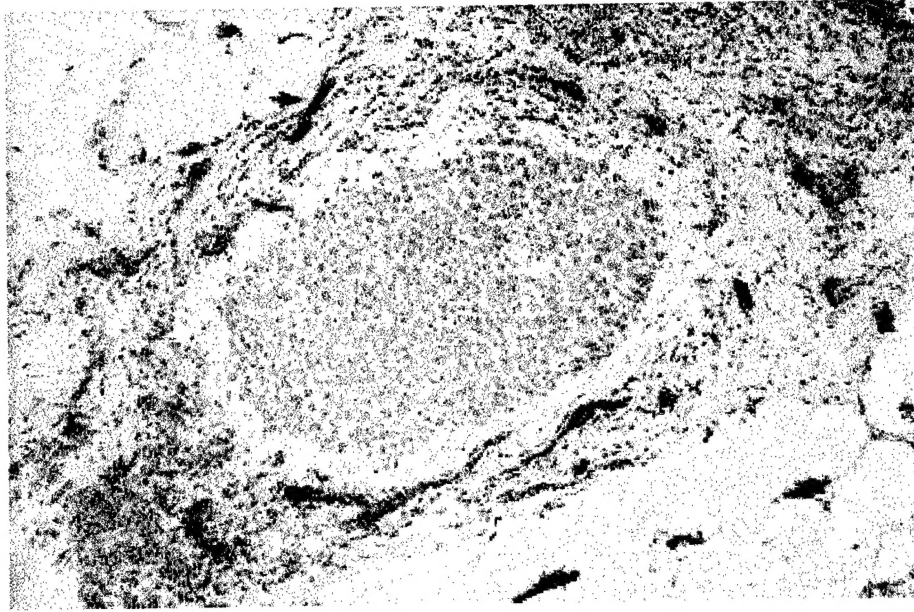


Figure 1. RT- PCR analysis of total RNA isolated from 19 breast cancer cell lines for Angiopoietin-1 with GAPDH as a loading control. Controls were in the form of water, Chinese hamster ovary (CHO) cells transfected with Angiopoietin 1 (CHO ANG-1) and CHO cells transfected with an empty vector (CHO- vector). The bottom two rows show PCR products for after RTPCR using ANG-1 and GAPDH primers, visualized with ethidium bromide staining. Arrows indicate PCR fragments of appropriate size. The GAPDH primers were designed around an intron to exclude DNA contamination, which would have yielded a larger PCR product. (The genomic sequence for Angiopoietin 1 is not known).

The top row is a southern hybridization of the Angiopoietin-1 PCR gel after transfer to a nitrocellulose membrane. The probe was a ^{32}P labeled oligonucleotide primer to a sequence of angiopoietin-1 between the PCR primers, confirming the PCR product to be angiopoietin-1. Unequivocal positive signals were seen in 8 out of 19 lines.

A



B

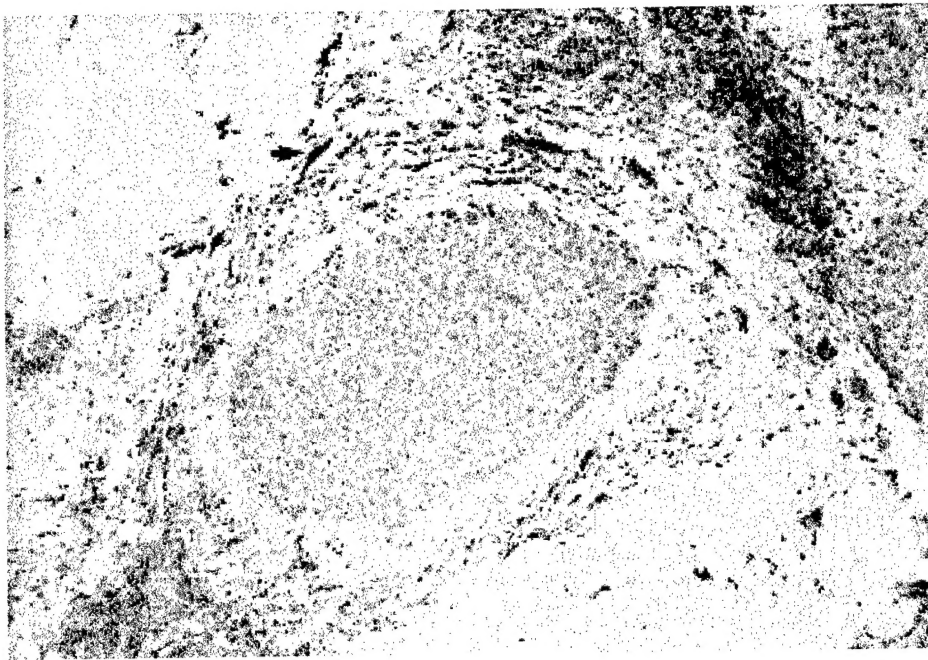
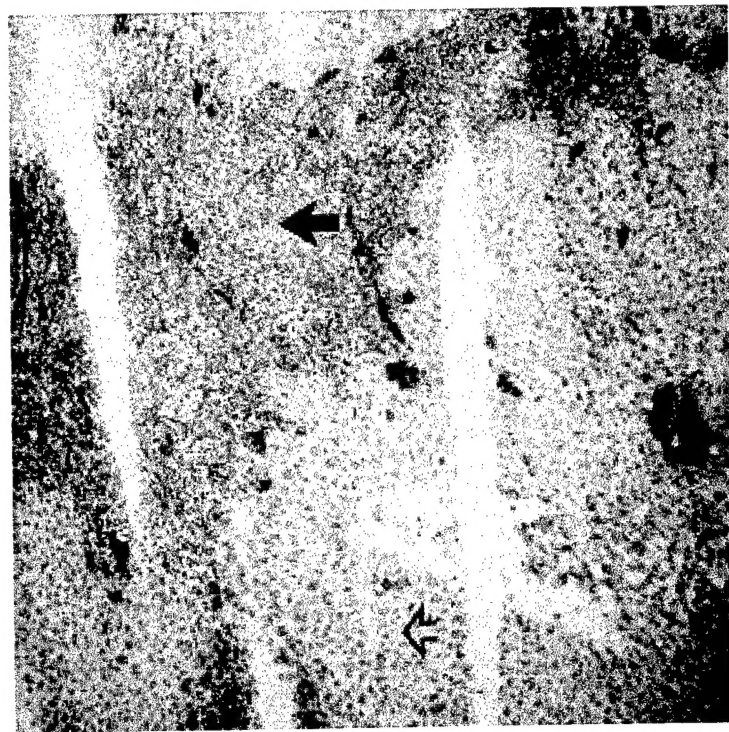


Figure 2. Immuno-staining in serial sections to Von Willebrand factor (A) and TIE-2 (B) in breast cancer tissue. Note the brown staining in vessels in both images in the stroma surrounding a duct full of cancer cells



A



B

Figure 3. Immuno-histochemistry to Von Willebrand factor (Panel A) and in-situ hybridization to ANG-1 (Panel B) in serial sections from of a lymph node containing metastatic breast cancer. Note in Panel A, dense areas of brown staining vessels in an area of lymphocytes outlined by the filled arrow. These vessels also stain to TIE-2 though the morphology is poorer. The larger cells are breast cancer cells outlined by the open arrow. Panel B is a dark field image of a serial section stained by in-situ hybridization to ANG-1. The bright silver granules (filled arrow) are positive signal to ANG-1 over breast cancer cells adjacent to the area of neo-vascularisation. Controls in the form of a sense probe or slides pre-treated by RNase digestion show no signal.

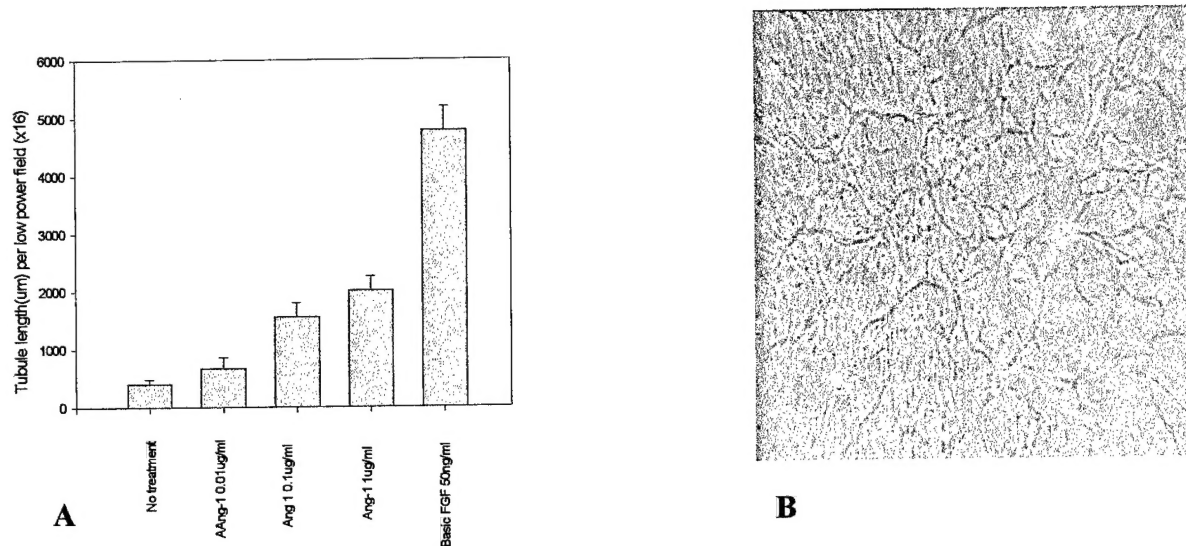


Figure 4. Panel A: A quantitative assessment of tubule formation by adult bovine aortic endothelial (ABAE) cells on collagen gels in response to increasing doses of ANG-1. ABAE cells are seeded on collagen gels in standard media and when confluent, treated with ANG-1, Basic fibroblast growth factor as positive control or media alone as a negative control. The tubules formed can be visualized by phase contrast microscopy (Panel B) and the tubule length can be quantitated using an Optimas® computerized image analysis system.

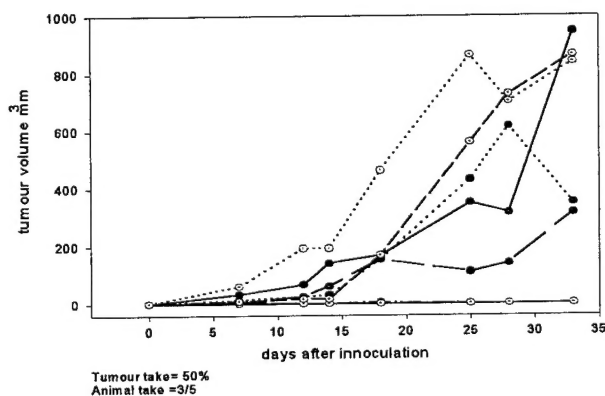


Figure 5 . Tumor growth curves for Chinese Hamster Ovary (CHO) cells transfected with the ANG-1 gene. Five athymic mice were injected with 5 million CHO cells bilaterally. The control in the form of an empty vector transfectant did not produce any tumor growth in any of ten injections. The *in vitro* growth characteristics of the ANG-1 transfected and the empty vector transfected CHO cells were identical

CONCLUSIONS

The goal of the proposed research is to investigate the possibility of using Tie-2, an endothelial cell-specific receptor tyrosine kinase, as a therapeutic target. The ligand of Tie-2 is the recently described novel factor named angiopoietin-1 (Ang-1). Ang-1 is vital to the development of an organized vasculature during embryonic development. We found earlier that Tie-2 is elevated in tumor neovasculature. We have prepared a fusion toxin consisting of Ang-1 and *Pseudomonas* exotoxin. Work is underway to examine the expected antiangiogenic and anticancer activity of the fusion toxin. In addition, we have investigated the expression of Ang-1 and Tie-2 in human breast cancer clinical specimen, and the possible function of Ang-1 as an angiogenic factor. Our data suggest that the Ang-1/Tie-2 system may play a key role in the angiogenic switch - conversion of cancer cells from a non-angiogenic phenotype to an angiogenic one. Insights into the molecular mechanisms involved will lead to the development of therapies that prevent the switch to an angiogenic phenotype and prevent progression from an in-situ to an invasive carcinoma. We will continue to examine the efficacy of the Ang-1-PE toxin for the treatment of breast cancer in animal models. We will continue to study the role of Ang-1/Tie-2 system in the angiogenic switch.

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